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Research Article

PKA and CDK5 can phosphorylate specific serines on the intracellular domain of podoplanin (PDPN) to inhibit cell motility

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ABSTRACT

Podoplanin (PDPN) is a transmembrane glycoprotein that promotes tumor cell migration, invasion, and cancer metastasis. In fact, PDPN expression is induced in many types of cancer. Thus, PDPN has emerged as a functionally relevant cancer biomarker and chemotherapeutic target. PDPN contains 2 intracellular serine residues that are conserved between species ranging from mouse to humans. Recent studies indicate that protein kinase A (PKA) can phosphorylate PDPN in order to inhibit cell migration. However, the number and identification of specific residues phosphorylated by PKA have not been defined. In addition, roles of other kinases that may phosphorylate PDPN to control cell migration have not been investigated. We report here that cyclin dependent kinase 5 (CDK5) can phosphorylate PDPN in addition to PKA. Moreover, results from this study indicate that PKA and CDK5 cooperate to phosphorylate PDPN on both intracellular serine residues to decrease cell motility. These results provide new insight into PDPN phosphorylation dynamics and the role of PDPN in cell motility. Understanding novel mechanisms of PDPN intracellular signaling could assist with designing novel targeted chemotherapeutic agents and procedures.

Abbreviations: PDPN, Podoplanin; PKA, Protein Kinase A; CDK5, Cyclin dependent Kinase 5

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Introduction

Podoplanin (PDPN) is a unique transmembrane receptor glycoprotein. Tumor promoters including TPA [1], RAS [2], TGF-β1, IFN-γ [3], EGFR [4], and Src [5] induce PDPN expression. PDPN regulates the activities of Rho, ezrin, and other proteins linked to the actin cytoskeleton to mediate filopodia formation, cell motility, invasion, and metastasis [6,7].

PDPN expression is critical for embryonic development, and is upregulated in many types of cancer [6,8–10]. For example, PDPN promotes the motility and invasion of many transformed cell types including mammary carcinoma [6,11], glioma [12], and oral squamous carcinoma cells [13–15]. PDPN is also expressed by lymphatic endothelial cells [16,17] and cancer associated fibroblasts [18–20] which can augment tumor invasion and metastasis [19,21,22].

PDPN can serve as a functionally relevant tumor biomarker, and is an enticing chemotherapeutic target. Indeed, the extracellular domain of PDPN can be targeted by antibodies and specific lectins to suppress tumor cell growth and migration [15,23–28]. However, signaling events mediated by the intracellular domain of PDPN may also illuminate mechanisms leading to cell motility and pathways to chemotherapy that have not been explored.

The PDPN intracellular domain consists of about 10 amino acids that direct interactions with ERM proteins to affect the actin cytoskeleton [29]. This domain contains 2 conserved serine residues that can be phosphorylated to decrease PDPN mediated cell migration [18]. However, specific kinases that phosphorylate these individual serine residues, and how these phosphorylation events affect cell migration, have not been clearly elucidated. Here, we present data indicating that PKA and CDK5 kinases cooperate to phosphorylate both serines in the intracellular tail of PDPN in order to decrease cell motility.

Materials and methods

Immunoprecipitation and Western blot analysis

Cells were lysed in RIPA buffer (50 mM Tris–HCl pH 7.4, 1% NP-40, 2.5 mM sodium pyrophosphate, 150 mM sodium chloride, 1 mm EDTA, 1 mM EGTA, 1 mM sodium betaglycerophosphate, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 1 mM PMSF, 10 μg/mL protease inhibitor cocktail) and clarified by centrifugation at 20000*g* for 5 min. Lysates containing 5 mg of protein were incubated with phosphoserine antibody 16B4 (Enzo ALX-804-167-C100) [30,31] for 3 h at 4 °C followed by protein-L beads (Santa Cruz 2336) for 1.5 h at 4 °C. Immune complexes were washed four times with ice-cold PBS supplemented with 1 mM sodium orthovanadate, 50 mM sodium fluoride, 1 mM PMSF, and 10 μg/mL protease inhibitor cocktail, eluted in sample buffer, resolved on 12% SDS-PAGE gels, and transferred to Immobilon-P membranes (Millipore IH1079562, Billerica, MA, USA). Western blotting was performed with antisera specific for PDPN [18], GAPDH (Santa Cruz Biotechnology A1978, Santa Cruz, CA, USA), or β-actin (Sigma A1978, St. Louis, MO, USA), and recognized by appropriate secondary antiserum conjugated to horseradish peroxidase and detected using enhanced chemiluminescence (Thermoscientific 32106, Philadelphia, PA, USA) as described previously [5,18,25]. Membranes were stained with India ink to verify equal loading and transfer after blotting.

Kinase assays

A phosphocellulose paper binding assay was used to measure phosphorylation of the peptide VVMKKISGRFSP (>95% purity, synthesized by GenScript USA Inc., Piscataway, NJ, USA), containing the entire intracellular region of PDPN (residues 161–172) as described [18]. Reaction mixtures contained 700 μM peptide in 20 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 1 mg/ml bovine serum albumin, 0.25 mM ATP, and [γ-^{32}P]–ATP (100 cpm/pmol). Reactions were initiated by adding 80 μg/ml PKA (Promega V5161, Madison, WI, USA), 22 μg/ml CDK5/p25 kinase enzyme (Promega V3231, Madison, WI, USA), or both PKA and CDK5/p25. Reactions were quenched after 10 min by the addition of 10% trichloroacetic acid, spotted onto P81 phosphocellulose filters, washed 3 times with 0.5% phosphoric acid, and analyzed by scintillation counting to determine picomoles of phosphate transferred. For mass spectrometry analysis, the kinase assay reactions contained 700 μM PDPN peptide in 30 mM Tris–HCl (pH 7.5), 20 mM $MgCl_2$, 1 mg/ml bovine serum albumin and 0.40 mM ATP.

Mass spectrometry

Peptide was purified (Millipore UFC501096 Carrigtwohill, Ireland) from kinase reactions and desalted (Millipore ZTC18M960, Billerica, MA, USA) according to manufacturer's instructions. Peptide was then subjected to LC-MS/MS analysis on an Ultimate 3000 liquid chromatography (LC) system coupled with an Orbitrap Velos tandem mass spectrometry (MS/MS) instrument (Thermo Fisher Scientific, Philadelphia, PA, USA). Resulting MS/MS spectra were searched against a database containing PDPN peptide sequence using a local Mascot search engine (V.2.3). Methionine oxidation and serine/threonine phosphorylation were set as variable modifications in search parameters. Phosphopeptide sequence and phosphorylation sites were manually confirmed based on y- and b-ion series fragments.

Generation of mutant PDPN cell lines

PDPN single mutant constructs were produced with the QuikChange II XL Site-Directed Mutagenesis Kit according to the manufacturer's protocol (Stratagene 200521, La Jolla, CA, USA). The vector pEF4Pdpn, encoding full length wild type mouse PDPN (PdpnWT) [18], was used as a template. PdpnSer167Ala, encoding PDPN with non-phosphorylatable serine at position 167 (PdpnS167A) was generated with the complementary primer pairs 5′GTTGTTATGAAGAAGATTGCTGGAAGGTTCTCGCC3′, 5′GGCGAGAACCTTCCAGCAATCTTCTTCATAACAAC3′ followed by 5′GGTTCTCGCCCTAAAGAGGGCCCTTCGAAGG3′, 5′CCTTCGAAGGGCCCTCTTTAGGGCGAGAACC3′. PdpnSer167Asp, encoding PDPN with serine 167 mutated to phosphomimetic aspartate (PdpnS167D), was generated with the complementary primers 5′GTTATGAAGAAGATTGATGGAAGGTTCTCGCCCTAAAGAGC3′, 5′GCTCTTTAGGGCGAGAACCTTCCATCAATCTTCTTCATAAC3′. PdpnSer171Ala, encoding PDPN with non-phosphorylatable serine at position 171 (PdpnS171A), was generated with the complementary primers 5′TGGAAGGTTCGCGCCCTAAAGAGGGCCCTTCGAAGG3′, 5′CCTTCGAAGGGCCCTCTTTAGGGCGCGAACCTTCCA3′. PdpnSer171Asp,

encoding PDPN with serine 171 mutated to phosphomimetic aspartate (PdpnS171D), was generated with the complementary primers 5′TGGAAGGTTCGACCCCTAAAGAGGGCCCTTCGAAGG3′, 5′CCTTCGAAGGGCCCTCTTTAGGGGTCGAACCTTCCA3′. All constructs were verified by sequencing. PdpnKo cells were stably transfected with PdpnS167A, PdpnS167D, PdpnS171A or PdpnS171D constructs and maintained in DMEM supplemented with 10% FBS as described [18]. Cells were cotransfected with PDPN constructs subcloned into pEF4Zeo, as well as pBabePuro, and selected for growth in both zeocin and puromycin, to achieve very high transfection efficiencies without taking clones as previously described [5,18,32–34]. Clones were not taken to minimize potential effects of clonal variation, and experiments were done in parallel to control for differences in serum effects and other environmental factors between experiments.

Evaluation of cell migration

500,000 cells were seeded in each well of 12-well cluster plates (CytoOne CC7682-7512, Ocala, FL), allowed to form a monolayer, scratched at the center to create a wound, and migration was quantitated as the number of cells that entered a field of $400 \times 400\ m^2$ in the center of the wound at 18 h as previously described [5,18,25]. Data were taken from three representative experiments ($n=3$) with 5 fields per experiment. For some experiments, cells were treated with 20 μM PKA inhibitor H-89 (Calbiochem 371963) or 0.1 μM CDK5 inhibitor Roscovitine (Calbiochem 557360) immediately after wounding.

Statistical analysis

Statistical analyses were performed using Prism (version 5, GraphPad Software).

Results

PKA and CDK5 phosphorylate the intracellular domain of PDPN

We generated cells from homozygous null PDPN knockout mice, called PdpnKo cells [18]. These cells serve as a clear foundation on which to examine the effects of specific PDPN isoforms and modifications on cell behavior. For example, as shown in Fig. 1a, PDPN can be immunoprecipitated from these cells with phosphoserine antiserum, and then detected by Western blotting to demonstrate that it is phosphorylated by serine kinases in vivo.

The intracellular domain of PDPN contains 2 conserved serine residues. These serines are located at residues 167 and 171 in mouse PDPN, corresponding to serines 157 and 161 in human PDPN. We thus sought to identify which of these serines is phosphorylated to affect PDPN signaling events.

Kinase specific phosphorylation sites on PDPN intracellular domain were predicted using the ExPASy NetPhosK server. These analyses identified PKA and CDK5 as ideal candidates for kinases that phosphorylate the intracellular serines of PDPN. Therefore, a peptide corresponding to the entire intracellular domain of PDPN was used as a substrate to evaluate the ability of PKA and CDK5 to phosphorylate these serine residues. As shown in Fig. 1b, PKA transferred about 60 pmol of phosphates to the PDPN peptide in 10 min. These data are consistent with previous studies indicating that PKA can phosphorylate PDPN intracellular serines [18].

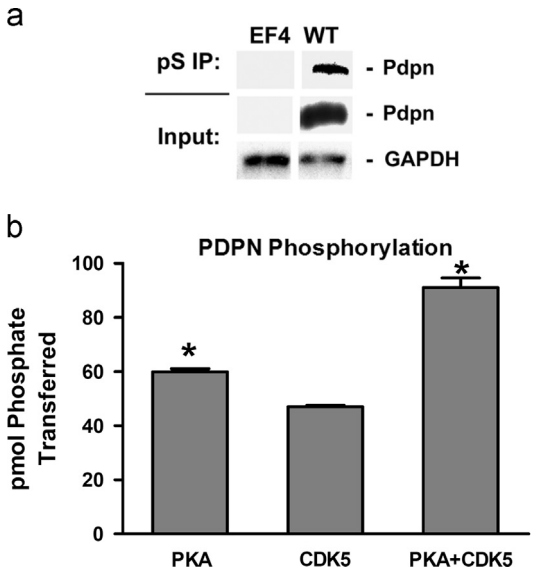

Fig. 1 – PKA and CDK5 phosphorylate serine residues in the PDPN intracellular domain. (a) Total protein (input) and protein immunoprecipitated with phosphoserine antiserum (pS IP) from homozygous null PdpnKo cells transfected with empty parental vector (EF4) or wild type PDPN (WT) was analyzed for PDPN and GAPDH by Western blotting as indicated. (b) Peptide containing the entire intracellular region (VVMKKISGRFSP) of PDPN was incubated with PKA, CDK5, or both PKA and CDK5 along with [γ-^{32}P] ATP for 10 min. Data are shown as picomoles of phosphate incorporated into the PDPN peptide (mean+SD, $n=2$). Asterisks indicate $p<0.05$ compared to CDK5 treated cells by *t*-Test.

In addition to PKA, CDK5 also transferred about 40 pmol of phosphate to the PDPN peptide in 10 min (Fig. 1b). Interestingly, simultaneous incubation with PKA and CDK5 resulted in approximately 100 pmol of phosphate to the PDPN peptide in 10 min (Fig. 1b). Thus, PKA and CDK5 displayed an additive effect on PDPN phosphorylation. Although serine 167 of PDPN has been considered to be a putative PKC phosphorylation site [13], we did not detect PDPN peptide phosphorylation by PKC (data not shown).

PKA phosphorylates PDPN on S167 or S171, while CDK5 preferentially phosphorylates S171

We performed LC-MS/MS analysis of the PDPN peptide from in vitro kinase assays in order to determine which PDPN residues are phosphorylated by PKA and CDK5. As shown in Fig. 2, treatment of the peptide with PKA or CDK5 resulted in singly phosphorylated species, in which either S167 or S171 was modified. MS spectra detected PDPN peptides with phosphorylation at serine 167 (S167) or serine 171 (S171) from PKA and CDK5 treated samples. We did not detect any PDPN peptides with phosphorylation at both S167 and S171 residues from these reactions (Figs. 2 and 3). These data indicate that each kinase can phosphorylate one or the other serine in a single reaction, but not both.

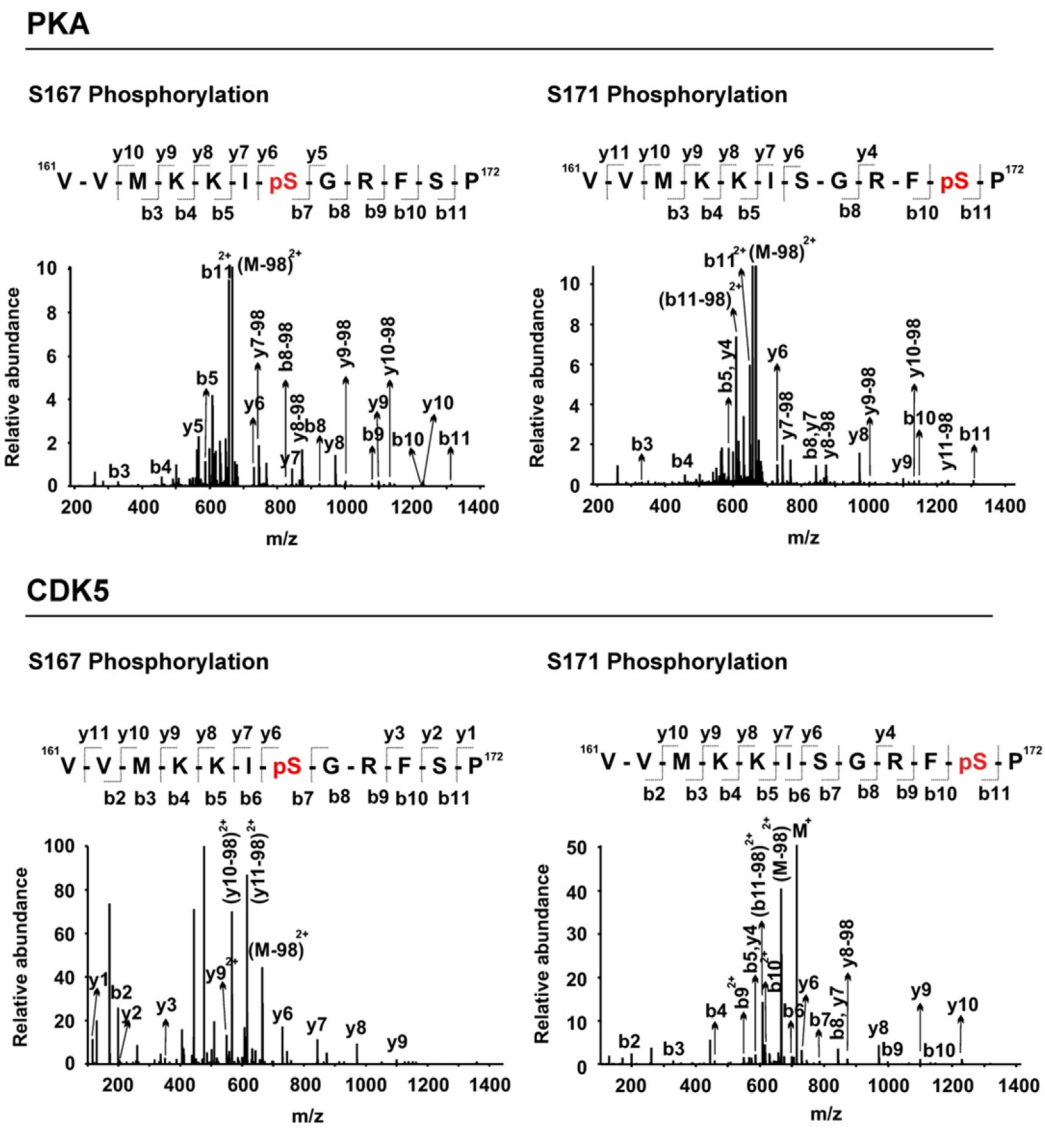

Fig. 2 – Peptide containing the entire intracellular region (VVMKKISGRFSP) of PDPN was incubated with PKA, CDK5, or both PKA and CDK5 along with [γ-^{32}P] ATP for 10 min. Representative MS/MS spectrum of PKA and CDK5 treated samples show doubly-charged ion (m/z 714.87) corresponding to the phosphorylated PDPN peptide sequence with phosphorylation at S167 or S171 as indicated. Observed y-ion and b-ion series confirming the peptide sequence and phosphorylation sites are also shown.

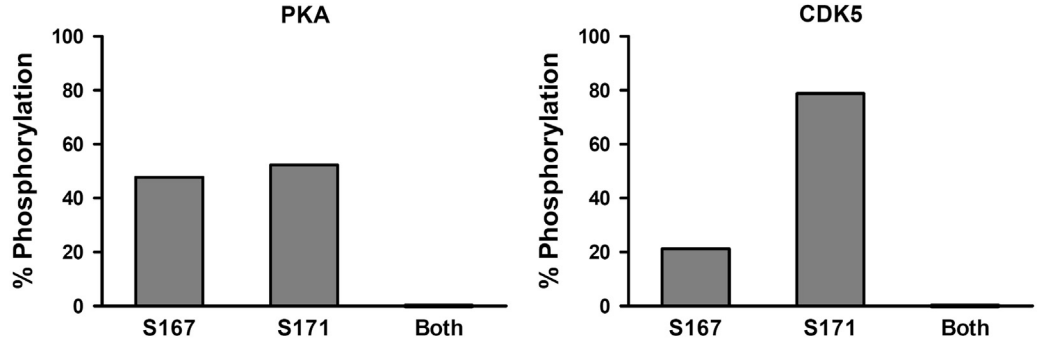

Fig. 3 – PKA phosphorylates S167 or S171, while CDK5 preferentially phosphorylates S171 in the PDPN intracellular domain. Peptide containing the entire PDPN intracellular region was treated with PKA or CDK5 as indicated, and analyzed by LC-MS/MS to detect specific phosphorylation sites. Data are shown as the percent of PDPN peptide phosphorylated at serine residues position 167 or 171 based on at least 100 MS spectra counts.

The percent of PDPN peptide phosphorylated at S167 or S171 was calculated from spectra counts to determine if PKA and CDK5 preferentially phosphorylate specific serine residues. As shown in Fig. 3, PKA phosphorylated PDPN on S167 or S171 equally well. However, CDK5 phosphorylated S171 four fold more than S167. These data indicate that PKA can phosphorylate PDPN equally well on either S167 or S171, whereas CDK5 preferentially phosphorylates PDPN on S171.

Both S167 and S171 residues on PDPN are phosphorylated to inhibit cell migration

We used homozygous null PDPN knockout mouse embryonic fibroblasts (PdpnKo cells) to evaluate how modification of each PDPN intracellular serine residue affects cell migration. As shown in Fig. 4b, cells expressing wild-type PDPN (PdpnWT) migrated about 2 fold more than control PdpnKo cells transfected with empty parental vectors (PdpnEF4). Substitution of both intracellular serines to nonphosphorylatable alanines (PdpnAA cells) increased cell migration about 3 fold compared to PdpnWT cells. In contrast to cells expressing nonphosphorylatable PDPN, migration of cells expressing PDPN with both serines mutated to phosphomimetic aspartate (PdpnDD) was comparable to control transfectants. These data are consistent with previous studies indicating that phosphorylation of PDPN can decrease cell migration [18].

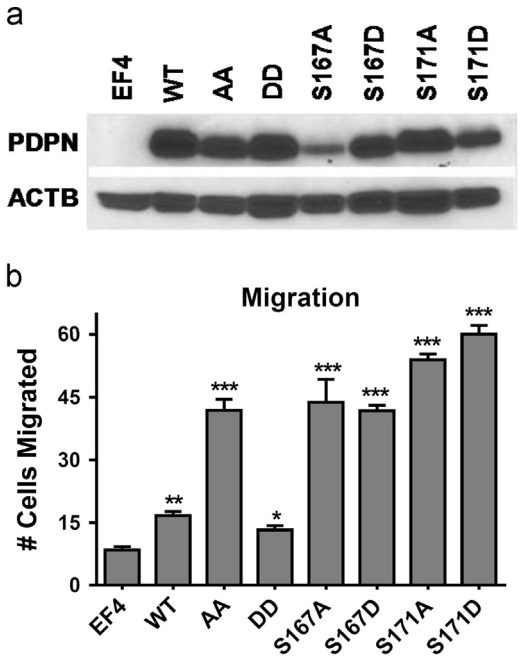

Fig. 4 – Both serine residues in the intracellular domain of PDPN are phosphorylated to decrease cell migration. (a) PDPN and β-actin were detected by Western Blotting of protein (10 μg/lane) from homozygous null Pdpn knock-out mouse embryonic fibroblasts transfected with empty parental vectors (EF4), wild type Pdpn (WT), Pdpn with both intracellular serines mutated to alanine (AA), Pdpn with both intracellular serines mutated to aspartate (DD), or Pdpn with each of the 2 serines mutated to alanine or aspartate (S167A, S167D, S171A, S171D) as indicated. (b) Cell migration was measured by wound healing assays, and quantitated as the number of cells that entered 400 × 400 μm field in the center of a wound within 18 h (mean+SEM, $n=3$). Single, double, and triple asterisks indicate $p<0.05$, 0.01, or 0.001 by t-Test compared to EF4 cells, respectively.

After examining double mutants, we generated cells expressing PDPN with single site mutations to examine the effects of phosphorylation of individual intracellular serine residues on cell migration. PdpnKo cells were transfected with PDPN constructs with S167 or S171 mutated to nonphosphorylatable alanine (PdpnS167A or PdpnS171A) and phosphomimetic constructs with these serines mutated to aspartate (PdpnS167D or PdpnS171D). After confirming PDPN expression by Western Blotting (Fig. 4a), cell migration was evaluated by wound healing assays.

As shown in Fig. 4b, cells expressing PDPN with either serine S167 or S171 mutated to alanine (PdpnS167A or Pdpn171A) migrated over twice as well as cells expressing wild type PDPN (PdpnWT). These data suggest that both of these serines need to be phosphorylated to effectively inhibit cell motility. As shown in Fig. 4b, cells expressing any construct with a single serine of PDPN mutated to alanine or aspartate migrated comparably to cells expressing nonphosphorylatable PDPN – with both sites mutated to alanine (PdpnAA). As discussed below, these data indicate that both intracellular serines on PDPN are phosphorylated to decrease cell migration, and phosphomimetic residues cannot fully compensate for these phosphorylation events.

Discussion

PDPN has emerged as a functionally relevant tumor biomarker and chemotherapeutic target [25,35,36]. Several ligands can bind to the extracellular region of PDPN to promote transformed cell migration and tumorigenesis [37–40]. Accordingly, this extracellular region can be targeted by reagents to inhibit tumor cell growth and motility [15,25,27,28].

Regardless of extracellular signals, the intracellular region of PDPN must also direct events that affect cell migration. The two conserved serine residues in the short PDPN intracellular tail present a clear potential for biological relevance. Results from studies presented here indicate that phosphorylation of either serine in the PDPN tail is not sufficient to inhibit cell migration; they both need to be phosphorylated to inhibit cell motility.

Interestingly, PdpnWT cells treated with a PKA inhibitor migrated about 50% more than control cells, while CDK5 inhibition decreased cell migration by about 50% (Supplemental Fig. 1). However, CDK5 regulates cell proliferation and morphology in addition to migration, and these pleiotropic effects may cause toxicities that prohibit cell motility in manners unrelated to PDPN signaling [41–43]. In addition, cells expressing phosphomimetic PDPN constructs with either intracellular serine mutated to aspartate (PdpnS167D or Pdpn171D) migrated twice as well as cells with wild type PDPN. Clearly, these phosphomimetic mutations do not reproduce PDPN modifications that result from phosphorylation events. These data are consistent with studies indicating that phosphate residues affect protein function in many ways, including alterations to potential adaptor binding sites and changes to the overall chemical environment that are not accomplished by changes of serines to aspartate or glutamate residues [44–46].

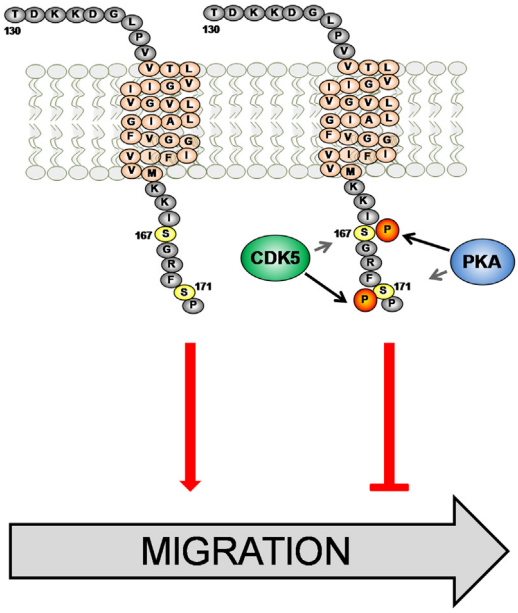

Fig. 5 – Phosphorylation of specific serines on the PDPN intracellular domain affect cell migration.
PKA can phosphorylate serines S167 or S171 on the intracellular domain of PDPN, whereas CDK5 preferentially phosphorylates S171. However, phosphorylation of both intracellular serines on PDPN is required to inhibit PDPN mediated cell migration. Thus, PKA and CDK5 may phosphorylate PDPN S167 and S171, respectively, in order to decrease cell motility.

Taken together, these results suggest a scenario, outlined in Fig. 5, in which PKA and CDK5 phosphorylate PDPN S167 or S171, respectively, in order to decrease cell motility. Previous studies found that PKA can phosphorylate PDPN on one or both intracellular serines to decrease cell migration [18]. Here, we show that both serines need to be phosphorylated in order to decrease cell migration. Moreover, these data indicate that PKA can phosphorylate PDPN on either serine, while CDK5 appears to phosphorylate S171. Therefore, a coordinated effort by unrelated kinases is required to phosphorylate PDPN in order to inhibit cell motility.

Identification of PKA and CDK5 as kinases that phosphorylate specific residues on PDPN to affect cell migration should help elucidate mechanisms that control processes including embryonic development, tumorigenesis, and chemotherapeutic treatments. For example, chemotherapeutic agents such as disulfiram and CARP-1 functional mimetics have recently been found to induce PDPN phosphorylation. Moreover, PDPN phosphorylation induced by these anticancer reagents leads to PDPN degradation and mesothelioma cell growth inhibition [30,31]. Further elucidation of how PDPN phosphorylation controls cell growth and motility should yield fundamental insights into many important biological and biomedical processes.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.yexcr.2015.04.019.

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